Ly6C^{hi} Monocytes Direct Alternatively Activated Profibrotic Macrophage Regulation of Lung Fibrosis

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***Rationale*: Idiopathic pulmonary fibrosis (IPF) is a devastating disease. Antiinflammatory therapies, including corticosteroids, are of no benefit. The role of monocytes and macrophages is therefore controversial.**

***Objectives*: To define the role of monocytes and macrophages during lung fibrogenesis and resolution, and explore the phenotype of the cells involved.**

***Methods*: We used multiple *in vivo* depletional strategies, backed up by adoptive transfer techniques. Further studies were performed on samples from patients with IPF.**

***Measurements and Main Results*: Depletion of lung macrophages during fibrogenesis reduced pulmonary fibrosis as measured by lung collagen ($P = 0.0079$); fibrosis score ($P = 0.0051$); and quantitative polymerase chain reaction for surrogate markers of fibrosis *Col1* ($P = 0.0083$) and *α-smooth muscle actin* ($P = 0.0349$). There was an associated reduction in markers of the profibrotic alternative macrophage activation phenotype, Ym1 ($P = 0.0179$), and Arginase1. The alternative macrophage marker CD163 was expressed on lung macrophages from patients with IPF. Depletion of Ly6C^{hi} circulating monocytes reduced pulmonary fibrosis ($P = 0.0052$) and the number of Ym1-positive alternatively activated lung macrophages ($P = 0.0310$). Their adoptive transfer during fibrogenesis exacerbated fibrosis ($P = 0.0304$); however, adoptively transferred CD45.1 Ly6C^{hi} cells were not found in the lungs of recipient CD45.2 mice.**

***Conclusions*: We demonstrate the importance of circulating monocytes and lung macrophages during pulmonary fibrosis, and emphasize the importance of the alternatively activated macrophage phenotype. We show that Ly6C^{hi} monocytes facilitate the progression of pulmonary fibrosis, but are not obviously engrafted into lungs thereafter. Finally, we provide empirical data to suggest that macrophages may have a resolution-promoting role during the reversible phase of bleomycin-induced pulmonary fibrosis.**

Keywords: immune cells; antiinflammatory; corticosteroids

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The role of monocytes or macrophages in the pathogenesis of pulmonary fibrosis is uncertain. It is believed that alternatively activated macrophages are profibrotic and induced by corticosteroids.

What This Study Adds to the Field

This study demonstrates that monocytes and macrophages are involved in the pathogenesis of lung fibrosis and defines the Ly6C^{hi} monocyte and alternatively activated macrophage phenotypes as key cellular subtypes. It highlights the potential importance of macrophages in fibrosis resolution.

Pulmonary fibrosis is the common end-point of many disorders of the lung, such as adult respiratory distress syndrome; chronic hypersensitivity pneumonitis; the idiopathic interstitial pneumonias; granulomatous disorders of the lung including sarcoidosis; occupational lung diseases, such as asbestosis and silicosis; collagen vascular diseases with lung involvement, such as rheumatoid arthritis, systemic sclerosis, and systemic lupus erythematosis; and lung infections, such as tuberculosis. The archetypal fibrotic lung disease, idiopathic pulmonary fibrosis (IPF), is a devastating disorder with an unknown natural history (1); there are no effective therapies (2), its incidence is rising (3), and it has a median survival of between 2.5 and 3.5 years (1).

There has been much controversy regarding the role of monocytes and macrophages in the pathogenesis of pulmonary fibrosis. Early studies suggested their importance (4–6), but the lack of benefit of corticosteroids in treating IPF (7) prompted a rethink. Researchers proposed that if antiinflammatory therapies did not help, then inflammation may be less important in the pathogenesis of the disease than was previously thought (8). A new hypothesis was developed in which aberrant wound healing was believed to be the more likely mechanism for disease development (9). Since then the available data on monocytes and macrophages have been interpreted by various authors as both emphasizing and minimizing their role. Several papers have recently suggested the importance of monocytes and macrophages (10, 11). Importantly, it has been demonstrated in rodents using the bleomycin model that lung macrophages could be the predominant source of transforming growth factor-β (TGF-β) (12), a most prominent fibrosis-inducing molecule. Furthermore, it has been

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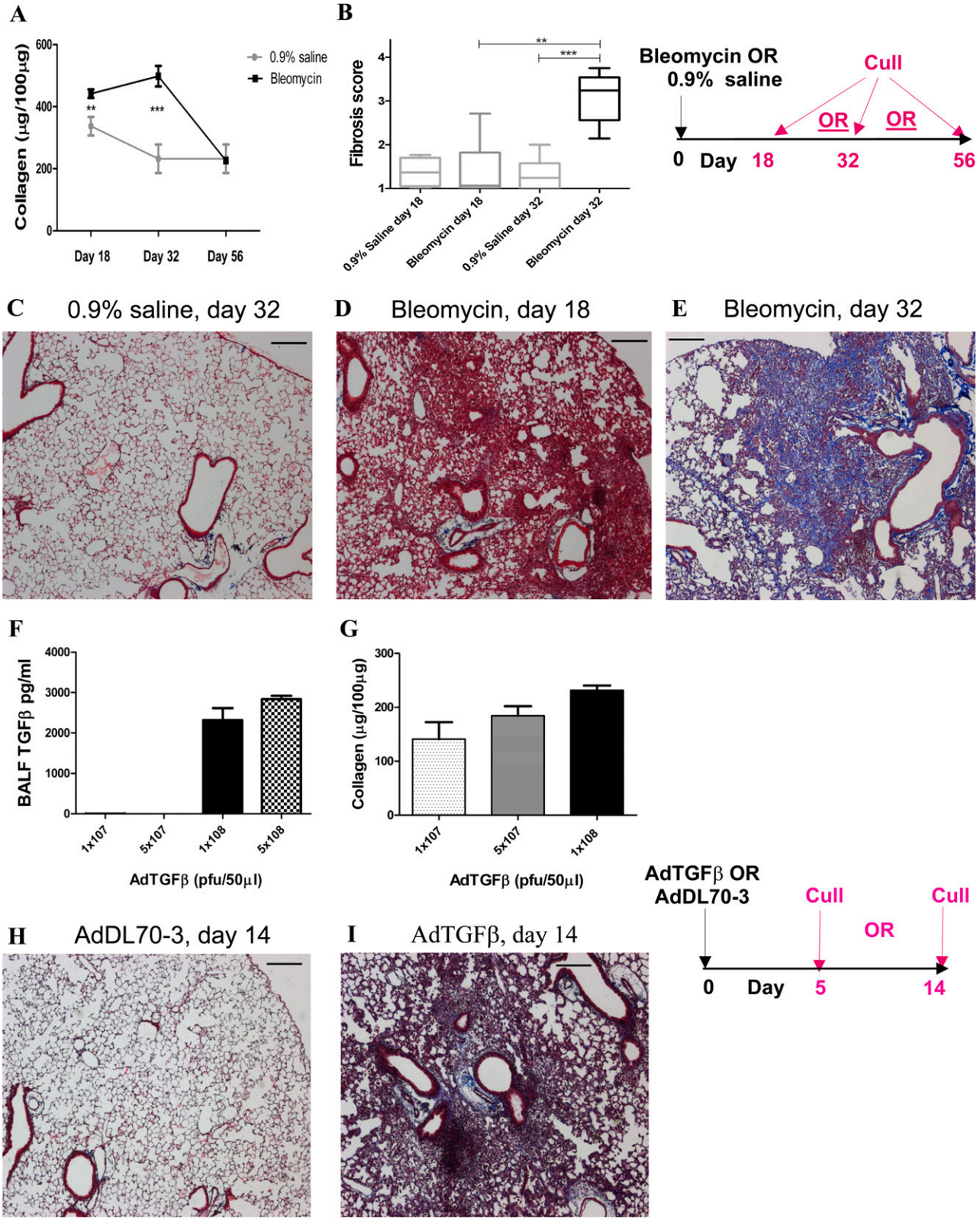
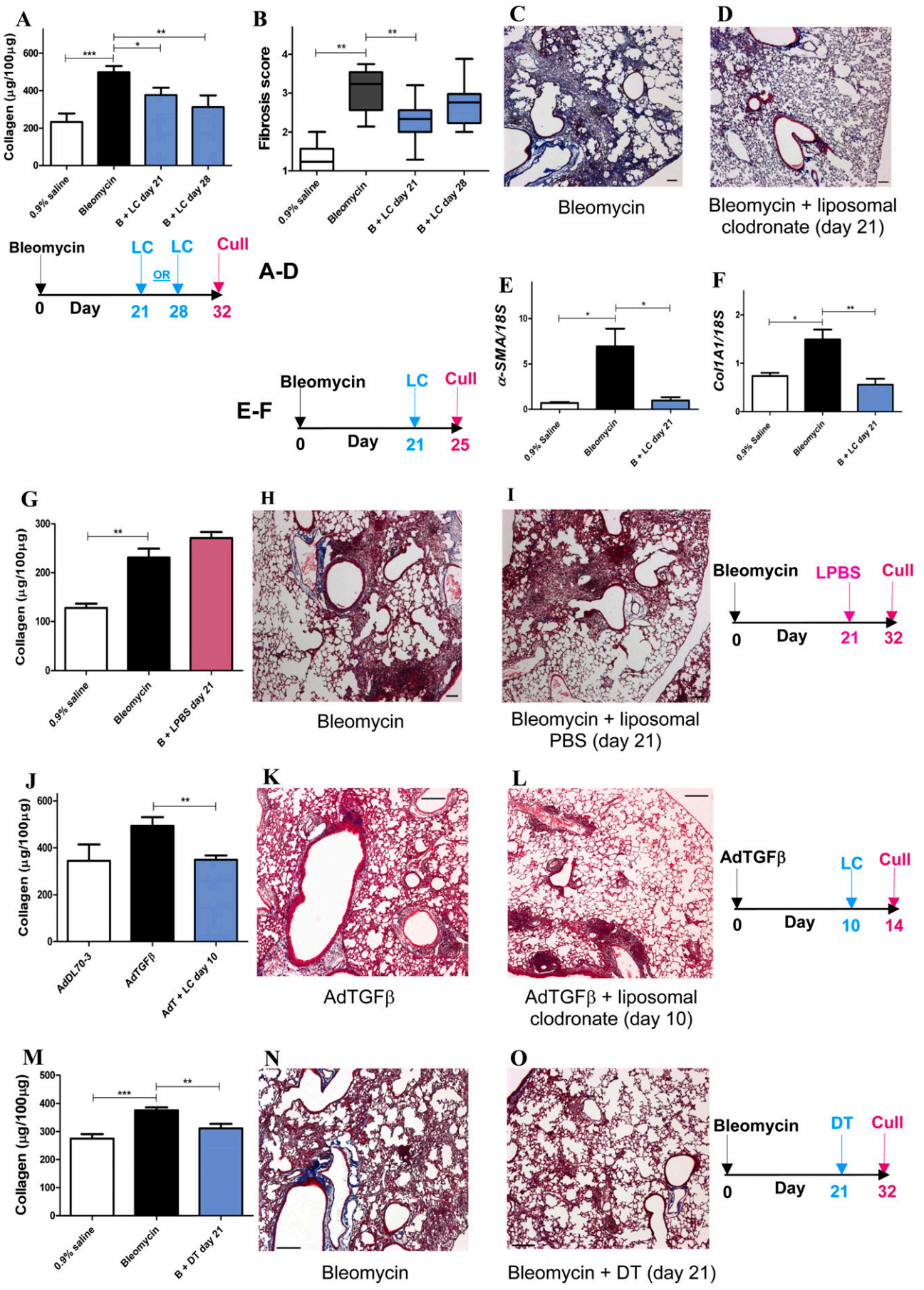


Figure 1. Bleomycin and adenoviral transforming growth factor-β (AdTGF-β) induce lung fibrosis. Female C57Bl/6 mice were given 0.05 U bleomycin (*A–E*) or 1×10^8 pfu AdTGF-β (*F–I*) intratracheally (50 μl). Saline 0.9% or empty viral vector (AdDL70–3) were given intratracheally as controls. Mice given bleomycin were culled during early (Day 18), progressive (Day 32), or resolving (Day 56) fibrosis. (*C–E*) Lung sections were stained with Masson's trichrome. Mice given AdTGF-β were culled at peak fibrosis (Day 14). Bronchoalveolar lavage fluid (BALF) TGF-β activity was measured after 5 days by ELISA (*F*) and lung fibrosis measured after 14 days by Sircol assay (*G*). (*H* and *I*) Lung sections were stained with Masson's trichrome. Data are presented as (*A*, *F*, and *G*) mean ± SEM, and (*B*) box and whiskers (median, interquartile range, minimum to maximum). (*A–E*) n = 4–8; (*F–I*) n = 3–5. *Bar* = 200 μm.


A
Collagen (µg/100µg)
0.9% saline
Bleomycin
B + LC day 21
B + LC day 28
B
Fibrosis score
C
Bleomycin
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Bleomycin
LC
LC
Cull
OR
0
Day
21
28
32
A-D
E-F
25
E
α-SMA/18S
F
Col1A1/18S
B + LC day 21
G
Collagen (µg/100µg)
B + LPBS day 21
H
Bleomycin
I
Bleomycin + liposomal PBS (day 21)
LPBS
J
Collagen (µg/100µg)
AdDL70-3
AdTGFβ
AdT + LC day 10
K
AdTGFβ
L
AdTGFβ + liposomal clodronate (day 10)
M
Collagen (µg/100µg)
B + DT day 21
N
Bleomycin
O
Bleomycin + DT (day 21)
DT

Figure 2. Lung macrophages regulate the fibrotic phase of pulmonary fibrosis. Female C57Bl/6 mice were given 0.05 U bleomycin (B; 50 μl) intratracheally on Day 0 ± liposomal clodronate (LC; 100 μl) intratracheally on Day 21 or Day 28, and culled on Day 32. (*A–D*) Depletion of macrophages reduces fibrosis as measured by collagen quantity (*A*) and fibrosis score (*B*). This is corroborated by Masson's trichrome staining, as shown in representative images (*C* and *D*). A further group of mice were given bleomycin on Day 0 ± liposomal clodronate on Day 21 and culled on Day 25. (*E* and *F*) Quantitative polymerase chain reaction analysis of lung homogenates showed a reduction in expression of α-*smooth muscle actin* (α-*SMA*) and *Col1A1* after depletion of lung macrophages, respectively. In all experiments 0.9% saline was given as an additional control. (*G–I*) As a further control, 0.05 U bleomycin was given intratracheally on Day 0 ± liposomal phosphate-buffered saline (LPBS; 100 μl) intratracheally on Day 21 and mice culled on Day 32. There was no reduction in lung fibrosis. (*J–L*) Female C57Bl/6 mice were given 1×10^8 pfu adenoviral transforming growth factor-β (AdTGF-β; 50 μl) intratracheally on Day 0 ± LC on Day 10 and culled on Day 14. AdDL70–3 was given intratracheally as a control. Depletion of lung macrophages reduced fibrosis as determined by collagen quantity (*J*), and corroborated by Masson's trichrome staining (*K* and *L*). (*M–O*) Female CD11c-DTR mice were given 0.05 U bleomycin (50 μl) intratracheally on Day 0 ± *Diphtheria* toxin (DT; 8 ng/g, 50 μl) intratracheally on Day 21 and culled on Day 32. Saline 0.9% was given as a control. Depletion of $CD11c^+$ cells reduced fibrosis as measured by collagen quantity (*M*), and corroborated by Masson's trichrome staining (*N* and *O*). Data are presented as mean ± SEM except (*B*); box and whiskers (median, interquartile range, minimum to maximum). Lung sections were stained with Masson's trichrome. (*A–D*) n = 6–15; (*E–O*) n = 4–7. (*C, D, H,* and *I*) *Bar* = 100 μm. (*K, L, N,* and *O*) *Bar* = 200 μm.

shown in a model of lung fibrosis that corticosteroid treatment has no effect on alveolar macrophage secretion of TGF-β, and that when alveolar macrophages from bleomycin-treated animals are cultured in the presence of corticosteroids there is in fact increased secretion of TGF-β (13). Moreover, a recent study in patients who develop an accelerated form of fibrotic lung disease demonstrated that they have dysregulated alveolar macrophages (14).

Alternatively activated macrophages are associated with fibrogenic phenotypes in mice and humans (15). In solid organs, such as the liver, the critical role of the macrophage in regulating fibrosis is well established (16). Crucially, corticosteroids have been shown to induce the alternatively activated, profibrotic macrophage phenotype (17). Here we categorically examine the role of circulating monocytes and lung macrophages in the pathogenesis of lung fibrosis, reaffirm the importance of the alternatively activated macrophage phenotype, and provide new evidence for the importance of the $Ly6C^{hi}$ monocyte phenotype. Finally, we provide preliminary insight into the potential importance of macrophages as mediators of lung fibrosis resolution. Some of the results of these studies have been previously reported in abstract form (18, 19).

METHODS

Animals

All procedures were performed in accordance with Home Office guidelines (Animals [Scientific Procedures] Act of 1986). Additional details are provided in the online supplement.

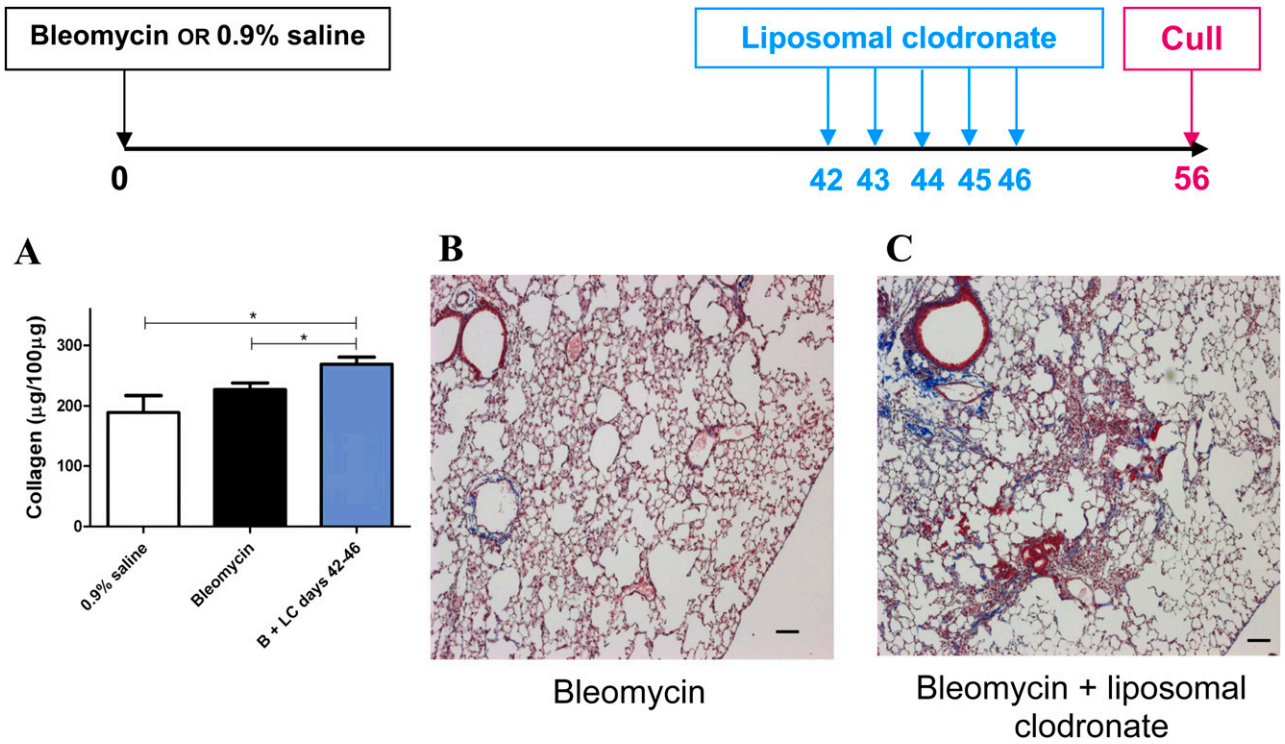
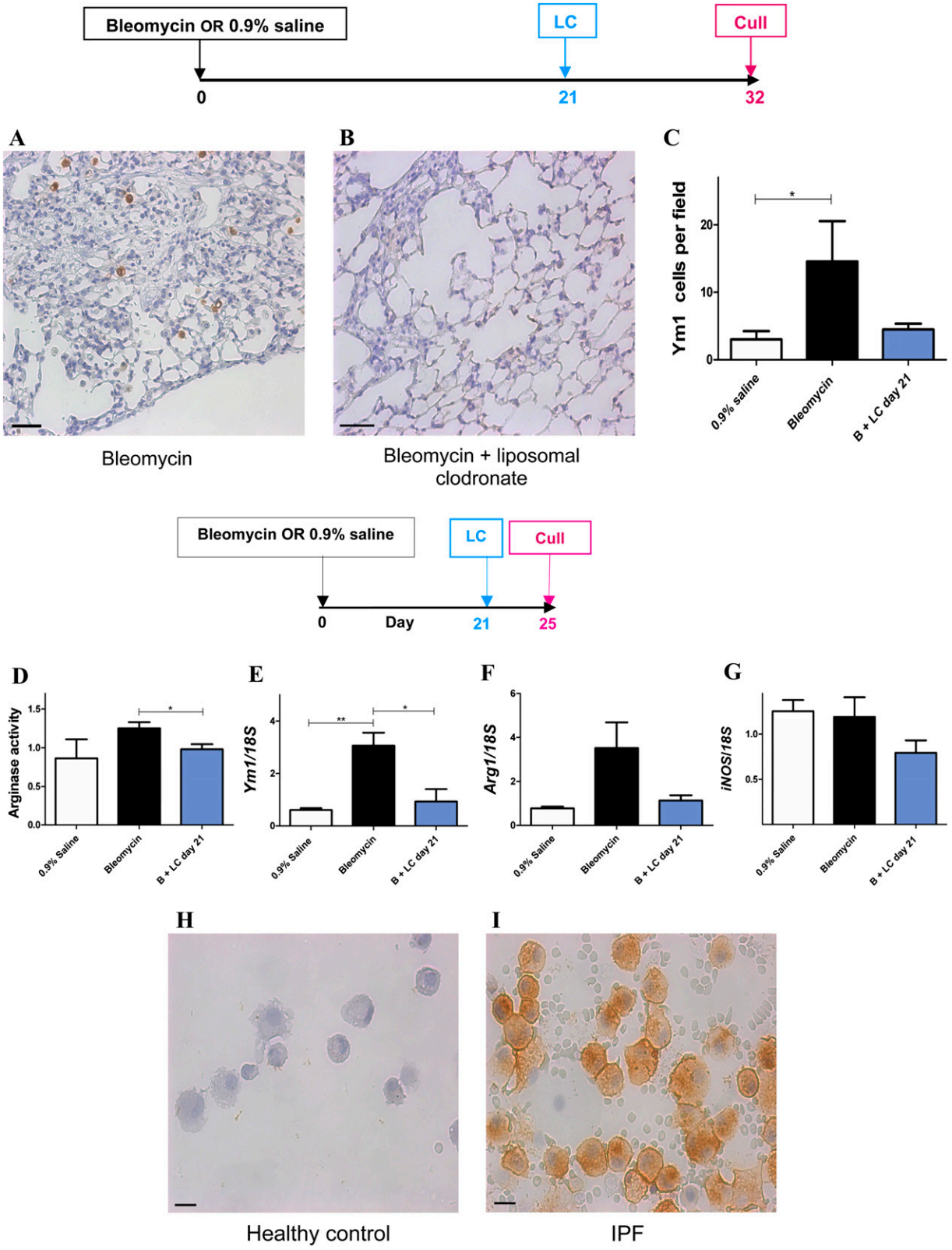


Figure 3. Lung macrophage depletion during recovery slows resolution of fibrosis. (*A–C*) Female C57Bl/6 mice were given 0.05 U bleomycin intratracheally (50 μl) on Day 0 ± liposomal clodronate (LC) intratracheally (100 μl) daily on Days 42–46 and culled on Day 56. Saline 0.9% was given intratracheally as a control. Depletion of lung macrophages slowed the resolution of fibrosis as determined by collagen quantity (*A*), and corroborated by Masson's trichrome staining (*B* and *C*). Representative images are shown. Data are presented as mean ± SEM. *Bar* = 100 μm. n = 6–7.



Bleomycin Model of Pulmonary Fibrosis

Female mice 10–14 weeks old and weight-matched were anesthetized with isoflurane (Merial, Harlow, UK). Bleomycin (0.025 or 0.05 U in 50 μl of 0.9% saline) (Apollo Scientific, Stockport, UK) or 0.9% saline was administered intratracheally. Clodronate was a gift of Roche Diagnostics (Burgess Hill, UK). It was encapsulated in liposomes as previously described (20). Liposomal phosphate-buffered saline was supplied by Nico van Rooijen (Amsterdam, The Netherlands). *Diphtheria* toxin (DT) was reconstituted in water, then diluted in phosphate-buffered saline (8 ng/g body weight). Mice were culled by intraperitoneal injection of pentobarbital (Merial). Tissue was obtained using standard techniques. Additional details are provided in the online supplement.

Figure 4. Alternatively activated macrophages characterize the progressive fibrotic phase of pulmonary fibrosis in mice and humans. (*A–C*) Female C57Bl/6 mice were given 0.05 U bleomycin (B) intratracheally on Day 0 ± liposomal clodronate (LC) intratracheally on Day 21 and culled on Day 32. Saline 0.9% was given intratracheally as a control. Lung sections were stained by immunohistochemistry for Ym1 (*A* and *B*), and quantified (*C*). Depletion of lung macrophages reduced the number of Ym1-positive cells (*C*). Images representative of staining are shown (*A* and *B*). (*D–G*) Further groups of mice were given bleomycin on Day 0 ± liposomal clodronate on Day 21 and culled on Day 25. Saline 0.9% was given intratracheally as control. Arginase activity (*D*) and quantitative polymerase chain reaction (*E–G*) were performed on lung homogenates. Depletion of lung macrophages decreased arginase activity (*D*), and decreased the expression of *Ym1* (*E*) and *Arginase 1* (*F*). There was no reduction in expression of *iNOS* (*G*). Cytospins were created from bronchoalveolar lavage fluid of age-matched healthy patients and patients with idiopathic pulmonary fibrosis (IPF). Slides were stained by immunocytochemistry for CD163. Macrophages from healthy patients do not stain for CD163 (*H*), but they do in patients with IPF (*I*). Data are presented as mean ± SEM. (*A–G*) n = 4–6; (*H* and *I*) n = 2–4. (*A* and *B*) *Bar* = 50 μm. (*H* and *I*) *Bar* = 20 μm.

Adenoviral TGF-β Model of Pulmonary Fibrosis

Female mice 10–14 weeks old, weight-matched, were anesthetized with isoflurane, and adenoviral (Ad) TGF-β or AdDL70–3 administered intratracheally. Additional details are provided in the online supplement.

Human Tissue Samples

Written informed consent was obtained. The study was approved by the Lothian Research Ethics Committee. Patients underwent a history, physical examination, high-resolution computed tomography, pulmonary function testing, bronchoalveolar lavage (BAL), and diagnostic lung biopsy where appropriate. All cases were discussed at the Lothian clinicopathoradiologic meeting and determined to have IPF by American Thoracic Society/European Respiratory Society criteria.

Determination of Lung Fibrosis

Paraffin-embedded sections of mouse tissue were stained with Masson's trichrome as per the manufacturer's instructions. Fibrosis score was evaluated (×100) as the quantity of the section positively stained for collagen and displaying alveolar wall thickening (1 = <25%, 2 = 25–50%, 3 = 50–75%, and 4 = 75–100%). Only fields where most of the field was composed of alveoli were scored. The entire lung section was analyzed. The investigator was masked to each sample.

Determination of Lung Collagen by Sircol Assay

Collagen content of the left lung was performed by Sircol assay as per the manufacturer's instructions (Biocolor, County Antrim, UK). Details are provided in the online supplement.

Arginase Assay

Arginase activity (per milligram of protein) in lung homogenates was assessed by the production of urea generated by the arginase-dependent hydrolysis of L-arginine as previously described (21).

Statistical Analyses

Paired data columns were evaluated using a two-tailed Student *t* test with GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA). Data were tested for normality and variance. Fibrosis score is presented as box and whiskers, interquartile range, minimum to maximum. Significance of the difference between medians was assessed using a Mann-Whitney test with GraphPad Prism. A P value less than 0.05 was considered significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Reagents, polymerase chain reaction (PCR) for TGF-β transgene and replication competent adenovirus (RCA) content of vector, immunohistochemistry, cytospins, immunocytochemistry, RNA extraction and quantitative PCR, flow cytometric analysis of lungs and blood, and flow sorting and adoptive transfer are provided in the online supplement.

RESULTS

Bleomycin and AdTGF-β Induce Pulmonary Fibrosis

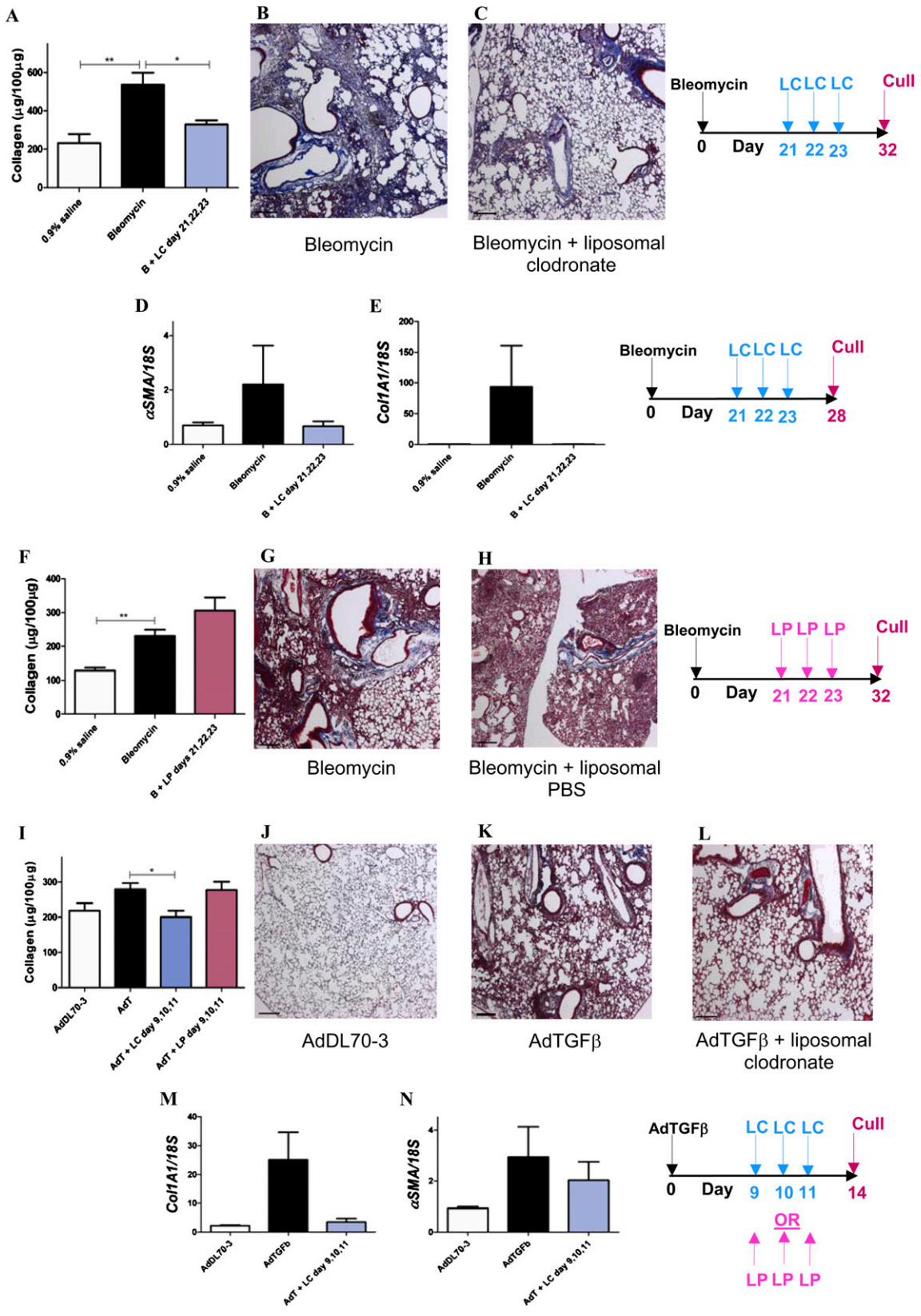
Administration of bleomycin by the intratracheal route induces initial inflammation and subsequent fibrosis. Fibrosis begins to develop after 14 days and usually peaks after 21–28 days (22). In our model administration of bleomycin (0.05 U) in female C57Bl/6 mice reliably causes acute inflammation over the first 10 days followed by early fibrosis at 18 days (Figures 1A, 1B, and 1D) and peak fibrosis at 32 days (Figures 1A, 1B, and 1E). Saline 0.9% does not induce fibrosis (Figures 1A–1C). Administration of AdTGF-β by the intratracheal route has been shown to induce prominent lung fibrosis with a peak at 14 days (23). In our model we established that at least 1×10^8 pfu/50 μl of AdTGF-β administered by the intratracheal route were required to ensure that TGF-β was detectable in BAL fluid (Figure 1F). Fibrosis had a dose-response (Figure 1G). At quantities of AdTGF-β of 5×10^8 pfu/50 μl mice experienced undue harm. Administration of empty viral vector (AdDL70–3) did not induce lung fibrosis (Figure 1H). The TGF-β vector was examined for content of transgene and RCA by PCR analysis (*see* Figure E1 in the online supplement). This confirmed that the wild-type adenovirus is positive for the presence of E1A (replication competent) and E2B (adenovirus backbone), and had no detectable TGF-β1 transgene. AdTGF-β vector is positive for E2B and TGF-β1 transgene, and had no detectable E1A, indicating that the RCA content is below the level of detection in the assay, reported to be below 1 part per 10^9 pfu of vector. This was confirmed using an independent cytopathic assay for RCA.

Liposomal Clodronate Depletes Lung Macrophages and DT Depletes CD11c$^+$ Cells in the CD11c-DTR Transgenic Mouse

Administration of liposomal clodronate is a well-established method of selectively and effectively depleting macrophages (24) including lung macrophages (20). Peak depletion occurs after 24 hours and persists for up to 5 days (20). We show that administration of liposomal clodronate (100 μl) by the intratracheal route effectively depletes macrophages in both naive (*see* Figures E2B and E2E) and bleomycin-treated mice (*see* Figures E2D and E2E). Administration of DT intratracheally to the CD11c-DTR transgenic mouse (25) is known to deplete lung CD11c$^+$ cells (26). In the lung interstitium, the major CD11c$^+$ cell type is the interstitial macrophage, dendritic cells being the minor component (26). Within the alveolar space alveolar macrophages represent 80–90% of the CD11c$^+$ cell population (26). Administration of DT depletes interstitial macrophages and dendritic cells for up to 9 days, and alveolar macrophages and dendritic cells for 2–4 days (26). Administration of DT intratracheally results in a greater than 90% depletion of CD11c$^+$ cells (*see* Figures E2G and E2H); large foamy macrophages are replaced by smaller mononuclear cells and occasional granulocytes (*see* Figures E2I and E2J).

Fibrosis is Independent of Monocytes and Lung Macrophages during the Inflammation Phase of Bleomycin Injury

Administration of liposomal clodronate during the inflammatory phase of bleomycin-induced lung injury leads to a significant



reduction in lung macrophages (*see* Figures E3A–E3C). We demonstrate that depletion of lung macrophages during the inflammatory phase of bleomycin injury has no effect on the early stage of lung fibrosis (*see* Figures E3D–E3F); this lack of effect is also seen when circulating monocytes are depleted (*see* Figures E3J–E3L). Furthermore, depletion of lung macrophages during the inflammatory phase of bleomycin-induced pulmonary fibrosis has no effect on peak fibrosis (*see* Figures E3G–E3I), and once more the same lack of effect is seen when circulating monocytes are depleted (*see* Figures E3M–E3O).

Figure 5. Circulating monocytes regulate the progressive fibrotic phase of pulmonary fibrosis in two independent murine models. (*A–C*) Female C57Bl/6 mice were given 0.05 U bleomycin (B) intratracheally on Day 0 ± liposomal clodronate (LC; 400 μl) intraperitoneally on Days 21, 22, and 23 and culled on Day 32. Saline 0.9% was given intratracheally as a control. Depletion of circulating monocytes decreased fibrosis as determined by collagen quantity (*A*), and corroborated by Masson's trichrome staining on lung sections. (*B* and *C*) Representative images are shown. (*D* and *E*) Further groups of mice were given bleomycin on Day 0 ± LC intraperitoneally on Days 21, 22, and 23 and culled on Day 28. Saline 0.9% was given intratracheally as a control. Quantitative polymerase chain reaction was performed on lung homogenates. Following depletion of circulating monocytes there was a reduction in expression of α-*smooth muscle actin* (α-*SMA*) (*D*) and *Col1A1* (*E*). (*F–H*) As a further control, groups of mice were given bleomycin on Day 0 ± liposomal phosphate-buffered saline (PBS) (LP) intraperitoneally on Days 21, 22, and 23 and culled on Day 32. Administration of LP had no effect on fibrosis, as determined by collagen quantity (*F*), and corroborated by Masson's trichrome staining as shown in representative images (*G* and *H*). Female C57Bl/6 mice were given 1×10^8 pfu adenoviral transforming growth factor-β (AdTGF-β) (AdT) or AdDL70–3 (control virus) intratracheally on Day 0 and culled on Day 14. In addition, in defined groups, liposomal clodronate (LC) or liposomal PBS (LP) were given intraperitoneally (400 μl) on Days 9, 10, and 11 (*I–N*). Depletion of circulating monocytes decreased fibrosis as determined by collagen quantity (*I*), and corroborated by Masson's trichrome staining, as shown in representative images (*J–L*). Administration of liposomal PBS had no effect on fibrosis (*I*). Depletion of circulating monocytes reduced expression of *Col1A1* (*M*) and α-*SMA* (*N*). Data are presented as mean ± SEM. n = 4–8. *Bar* = 200 μm.

Lung Macrophages Regulate the Progressive Fibrotic Phase of Pulmonary Fibrosis

Next we wished to evaluate the role of lung macrophages during the progressive fibrotic phase of pulmonary fibrosis. We show that depletion of lung macrophages during this phase of bleomycin injury significantly reduces the degree of pulmonary fibrosis (Figures 2A–2D). This is corroborated by a reduction in expression of the surrogate markers of fibrosis α-*smooth muscle actin* (α-*SMA*) and *Col1A1* (Figures 2E and 2F, respectively). It has previously been shown that the effect (of liposomal clodronate) is not caused by apoptosis *per se* because rats given apoptotic macrophages (27) or apoptotic cells (28) actually develop lung fibrosis. Furthermore, in an independent experiment we demonstrate that the effect is not merely a liposomal effect (Figures 2G–2I).

Because the role of monocytes and macrophages in lung fibrosis has been debated for years, we wished to back up our findings using a second model. Using the AdTGF-β model we show that depletion of lung macrophages once again led to a reduction in pulmonary fibrosis (Figures 2J–2L).

For categorical confirmation, we assessed the effect of depletion of lung macrophages on the degree of lung fibrosis using the bleomycin model but using an independent and specific conditional system, that of the CD11c-DTR transgenic mouse (25), and importantly we demonstrate that depletion of lung macrophages results in a significant reduction in lung fibrosis (Figures 2M–2O). It should be noted that this system also depletes lung dendritic cells (see Discussion).

Lung Macrophage Depletion during Recovery Slows Resolution of Fibrosis

Interestingly, depletion of lung macrophages during the resolution phase of bleomycin-induced lung fibrosis has the opposite effect (compared with fibrogenesis), slowing the resolution of fibrosis (Figures 3A–3C). This novel finding is in keeping with the role of macrophages during the resolution of liver fibrosis (16) and fits nicely with work showing that alveolar macrophages are involved in the removal of accumulated collagen (29), suggesting that macrophage manipulation for modulation of fibrosis may be possible.

Alternatively Activated Lung Macrophages Characterize the Progressive Fibrotic Phase of Pulmonary Fibrosis in Mice

Bleomycin administration increases the number of Ym1-positive lung macrophages compared with 0.9% saline control (Figures 4A–4C). In mice, Ym1 and arginase 1 are markers of alternative macrophage activation (30). Interestingly, we demonstrate that depletion of lung macrophages leads to a reduction in Ym1 protein expression (Figures 4A–4C) and arginase activity (Figure 4D). This effect of reduction in markers of alternative macrophage activation after macrophage depletion was confirmed at the RNA level (Figures 4E and 4F). There was no significant reduction in the gene expression of *iNOS*, the prototypical marker of classical macrophage activation (Figure 4G). These data suggest that it may be the alternatively activated lung macrophage that regulates the progressive phase of lung fibrosis.

Alternatively Activated Lung Macrophages Are Present in Patients with IPF

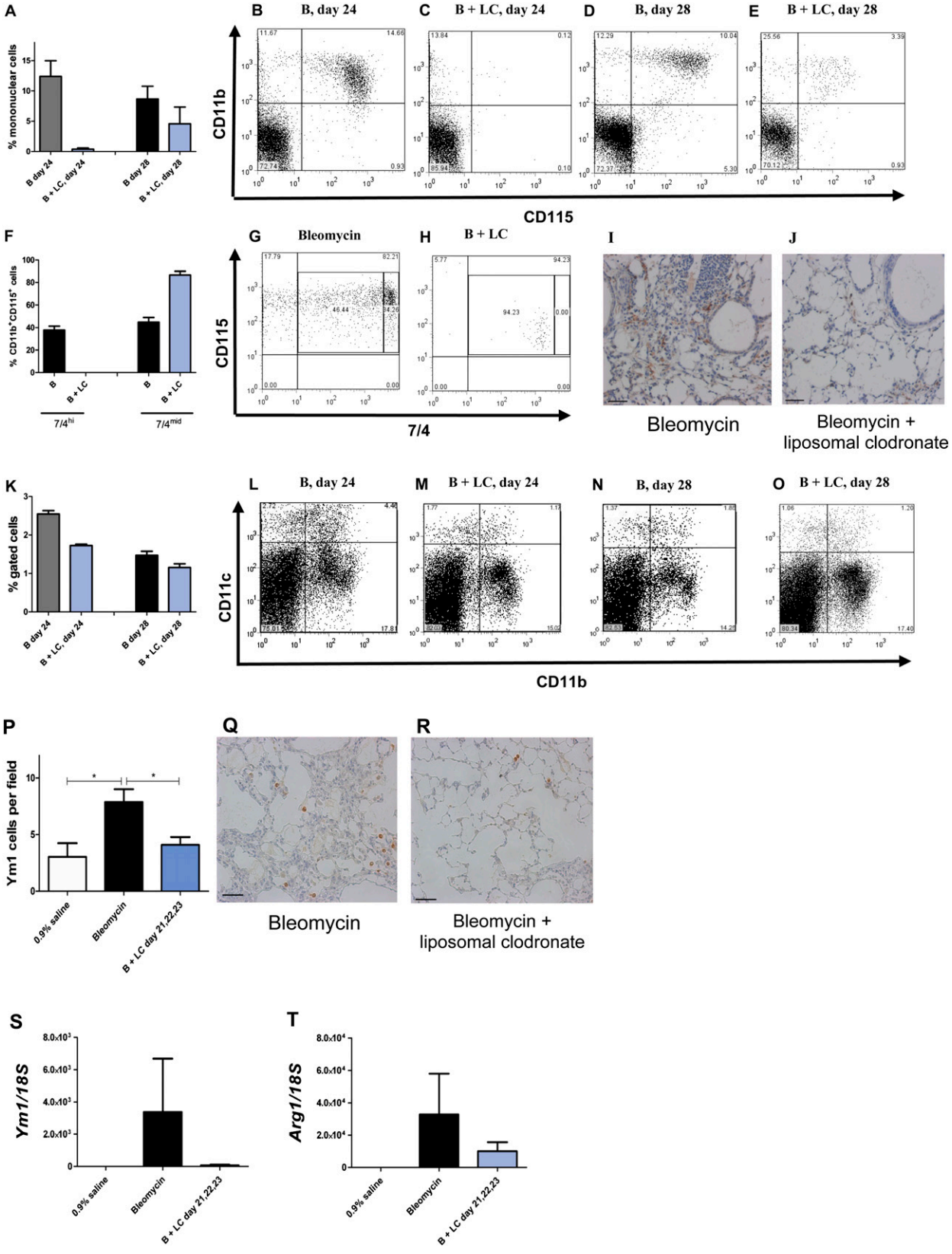
Having suggested that alternatively activated lung macrophages are important during the progressive phase of fibrosis in a murine model of pulmonary fibrosis we wished to establish whether alternatively activated macrophages are present in human disease. CD163, a human marker of alternative macrophage activation (31), was not expressed on lung macrophages from elderly healthy volunteers (Figure 4H), but importantly and in contrast, was shown to be expressed on greater than 90% of lung macrophages from patients with IPF (Figure 4I).

Circulating Monocytes Regulate the Progressive Fibrotic Phase of Pulmonary Fibrosis

Having established that lung macrophages regulate the progressive fibrotic phase of pulmonary fibrosis, we wished to establish whether circulating monocytes are also important, and whether there is a link between them. Depletion of circulating monocytes during the progressive fibrotic phase significantly reduces the degree of pulmonary fibrosis that develops (Figures 5A–5C). This is corroborated by quantitative PCR analysis of the surrogate markers of lung fibrosis, α-*SMA* and *Col1A1* (Figures 5D and 5E, respectively), and in an independent experiment we demonstrate that this effect was caused by circulating monocyte depletion *per se* rather then being a liposomal effect (Figures 5F–5H). Crucially, using the AdTGF-β model we found the same effects (Figures 5I–5N).

"Inflammatory" Circulating Monocyte Depletion Affects Alternatively Activated Lung Macrophages

To establish the subsequent effects of depletion of circulating monocytes on alternatively activated lung macrophages, we determined the effect of circulating monocyte depletion on lung macrophage populations and analyzed whether a particular monocyte subset was of particular importance. Circulating monocytes are $CD11b^+$, $CD115^+$ (32). We confirmed by flow cytometry of whole blood that administration of liposomal



clodronate (400 μl) intraperitoneally effectively depletes circulating monocytes (Figures 6A–6C). By 5 days after the final injection of liposomal clodronate the numbers of circulating monocytes remain reduced, but are approaching control levels (Figures 6A, 6D, and 6E).

Circulating monocytes have been subdivided into two major subsets. The "inflammatory" subgroup has been defined as $7/4^{hi}$, $Ly6C^{hi}$,$CCR2^{+}$,CX_3CR1^{-} and the second subgroup has been defined as $7/4^{mid}$,$Ly6C^{lo}$,$CCR2^{-}$,CX_3CR1^{+} (32–34). We show that in the context of bleomycin-induced pulmonary fibrosis,

Figure 6. "Inflammatory" monocyte depletion affects alternatively activated lung macrophages. Female C57Bl/6 mice were given 0.05 U bleomycin (B) intratracheally on Day 0 ± liposomal clodronate (LC) intraperitoneally on Days 21, 22, and 23 and culled on Day 24 (*A–C, F–H,* and *K–M*), Day 25 (*I, J, S,* and *T*), or Day 28 (*A, D, E, K,* and *N–R*). Blood (*A–H*) and lungs (*K–O*) were analyzed by flow cytometry. Immunohistochemistry was performed on lung sections for F4/80 (*I* and *J*) and Ym1 (*P–R*). Quantitative polymerase chain reaction was performed on lung homogenates (*S* and *T*). Administration of LC effectively depletes $CD115^+$, $CD11b^+$ monocytes 24 hours after the final injection (*A* and *C*) compared with control (*A* and *B*). By 5 days after the final injection of LC numbers of $CD115^+$, $CD11b^+$ monocytes remain reduced (*A* and *E*) compared with control (*A* and *D*), but are beginning to return to the circulation. In the context of bleomycin injury, administration of LC preferentially and effectively depletes "inflammatory" $CD115^+$, $7/4^{hi}$ monocytes (*F* and *H*) compared with control (*F* and *G*). Depletion of circulating monocytes reduces the number of F4/80-stained lung macrophages (*J*) compared with control (*I*). This is corroborated by flow cytometry of lung homogenates were depletion of circulating monocytes reduces the proportion of mononuclear phagocytes (*K* and *M*) compared with control (*K* and *L*). By 5 days after the final injection of LC, the proportion of lung mononuclear phagocytes (*K* and *O*) are approaching those of control (*K* and *N*). Furthermore, depletion of circulating monocytes decreases the number of Ym1-positive cells in the lung (*P*). This is shown in representative images (*Q* and *R*). Moreover, depletion of circulating monocytes reduces the expression (in the lung) of *Ym1* (*S*) and *Arginase1* (*T*). Data are presented as mean ± SEM. (*A–H* and *K–O*) n = 3; (*I* and *J*) n = 5; (*P–T*) n = 4–6. *Bar* = 200 μm.

intraperitoneal liposomal clodronate effectively depletes the "inflammatory" monocyte subgroup (Figures 6F–6H). Depletion of circulating monocytes is followed by a reduction in the lung macrophage population at 24 hours (Figures 6K–6M) and 48 hours (Figures 6I and 6J); after a further 4 days the lung macrophage proportion remains slightly reduced but is approaching control proportions (Figures 6K, 6N, and 6O). Interestingly, we demonstrate that the depletion of circulating monocytes is followed by a reduction in alternatively activated lung macrophage markers at both the protein (Figures 6P–6R) and RNA levels (Figures 6S and 6T). These findings demonstrate that depletion of circulating monocytes affects lung macrophage populations.

Adoptive Transfer of $Ly6C^{hi}$ "Inflammatory" Monocyte Progenitor Cells Exacerbates Pulmonary Fibrosis

Having identified that depletion of circulating monocytes seems to preferentially affect the "inflammatory" monocyte subgroup, we wished to establish categorically whether this monocyte subgroup is indeed important in promoting pulmonary fibrosis by effects mediated during the progressive fibrotic phase. Female C57Bl/6 mice were given a lower dose of bleomycin intratracheally (0.025 U) followed by adoptive transfer of 1×10^6 bone marrow–derived, flow-sorted (Figures 7A–7C), $Ly6C^{hi}$ "inflammatory" monocyte progenitor cells (by intravenous injection) during the progressive fibrotic phase (control mice were injected with media alone or $Ly6C^-$, $CD11b^-$ cells). We show that this significantly exacerbates pulmonary fibrosis (Figures 7D–7F), demonstrating that the $Ly6C^{hi}$ "inflammatory" monocyte population is indeed important in promoting pulmonary fibrosis via effects mediated during this period. Administration of the $Ly6C^{hi}$ "inflammatory" monocyte progenitor cell population is followed by an increase in the number of Ym1-positive alternatively activated lung macrophages compared with control subjects (Figures 7G–7I). We therefore wished to ascertain whether the $Ly6C^{hi}$ "inflammatory" monocytes were the direct precursor of the alternatively activated lung macrophages. $CD45.1^+$ bone marrow–derived $Ly6C^{hi}$ monocyte progenitor cells were adoptively transferred into CD45.2 bleomycin-treated mice during the progressive fibrotic phase (Day 21); strikingly, no CD45.1-derived cells were found in the lungs 24 hours after transfer (Figures 7M and 7N). Similarly, no cells were found 72 hours after transfer (data not shown). $Ly6C^{hi}$ mononuclear phagocytes were, however, found in the lungs during this progressive fibrotic period, and their phenotype is more in keeping with lung macrophages ($CD11c^+$, MHC II^{lo}) than dendritic cells ($CD11c^+$, MHC II^{hi}) (Figures 7J–7L).

DISCUSSION

We have demonstrated that lung macrophages do not seem to be involved in the early inflammatory phase of bleomycin-induced lung fibrosis and, in addition, that their presence during this phase is not crucial for the subsequent development of progressive pulmonary fibrosis. However, we show using two independent macrophage-depletional strategies in the bleomycin model that they are indeed important during the subsequent progressive fibrotic phase, the disease type that more closely resembles that seen in humans, and define the importance of the alternatively activated lung macrophage phenotype. Moreover, we demonstrate the importance of lung macrophages in the adenoviral TGF-β–mediated model of progressive lung fibrosis. The TGF-β vector was shown to be RCA free and the sequelae seen after administration can therefore be assumed to be caused by overexpression of the TGF-β1 transgene. TGF-β is widely recognized to be a "master" molecule during the development of lung fibrosis (35) and the fact that lung macrophage modulation limits induced lung fibrosis emphasizes their importance. It is likely that by using the CD11c-DTR mouse there is an inherent depletion of lung dendritic cells, and their potential importance in the pathogenesis of pulmonary fibrosis has previously been highlighted (36). It is important to state that some experts believe that dendritic cells are a subgroup of macrophages (37, 38). The cell surface markers CD11c, CD11b, and MHC II have been used in varying combinations to define lung macrophages and dendritic cells (26, 36–39). In the resting state it has been suggested that lung macrophages are $CD11c^+$, $CD11b^-$, MHC II^{lo} and dendritic cells $CD11c^+$, $CD11b^+$, MHC II^+ (26, 39). However, it has been demonstrated that in the context of lung injury lung macrophages can be $CD11c^+$, $CD11b^+$ and dendritic cells $CD11c^+$, $CD11b^+$ but with a relative reduction in CD11b (40). It is apparent that the cell surface markers used to define macrophages and dendritic cells may lead to inherent confusion (37, 38). It is important not to minimize the role of dendritic cells in the pathogenesis of pulmonary fibrosis, although at the same time recognizing that there may be much more overlap in lung macrophage and dendritic cell definition and functionality than was previously thought (37, 38), and that current strategies used to define these cells may be suboptimal.

Our study demonstrates the novel finding that intratracheal instillation of bleomycin induces up-regulation of the alternative macrophage markers Ym1 and arginase1, and that after lung macrophage depletion, gene expression and protein levels are reduced for both of these markers. Ym1, also called chitinase 3–like protein 3, is an archetypal murine marker of alternatively activated macrophages (30). The function of Ym1 is uncertain. Recent work, however, has suggested that it is a marker of oxidative stress, known to be important in the pathogenesis of lung fibrosis (41). No human homolog has yet been identified; however, YKL-40 (also called chitinase 3–like protein 1), a human member of the same chitinase family of proteins, has been found to be elevated in the circulation (42) and in liver tissue of patients with

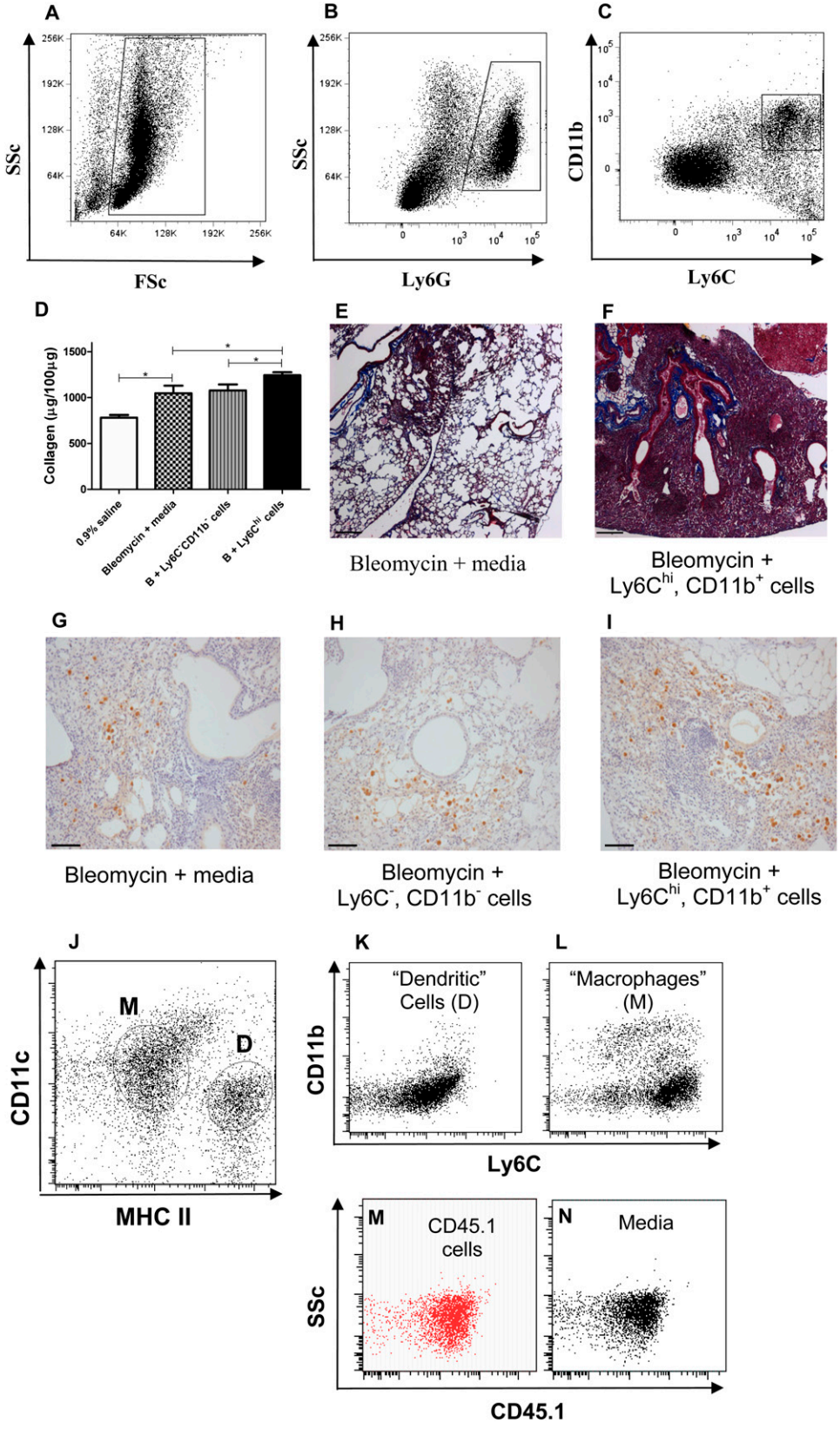


Figure 7. Adoptive transfer of "inflammatory" Ly6C^{hi} monocyte progenitor cells exacerbates pulmonary fibrosis in the bleomycin model. Female C57Bl/6 mice were given a reduced dose of bleomycin (0.025 U), (B), intratracheally (50 μl) on Day 0 plus 1×10^6 bone marrow-derived Ly6C^{hi} monocyte progenitor cells intravenously (tail vein) on Day 21 and culled on Day 28. Controls of media or Ly6C$^-$,CD11b$^-$ cells were given intravenously on Day 21 (*A–I*). In another experiment bone marrow–derived Ly6C^{hi} monocyte progenitor cells from female C57Bl/6 CD45.1 mice were injected intravenously into CD45.2 mice (pretreated with bleomycin 0.05 U) on Day 21, and mice culled on Day 22 (*J–N*). Ly6C^{hi} monocyte progenitor cells were sorted from bone marrow as follows: live cells were gated (*A*), then Ly6G$^+$ granulocytes positively excluded (*B*). From the resulting negative fraction, Ly6C^{hi}, CD11b$^+$ monocyte progenitor cells were positively sorted (*C*). Administration of Ly6^{hi} monocyte progenitor cells exacerbates fibrosis as determined by collagen content (*D*), and corroborated by Masson's trichrome staining of lung sections, as shown in representative images (*E* and *F*). Lung sections were also stained by immunohistochemistry for Ym1 (*G–I*). As shown in representative images, administration of Ly6C^{hi} monocyte progenitor cells leads to an increase in the proportion of Ym1-positive cells in the lung (*I*) compared with controls (*G* and *H*). Lung homogenates (from mice culled 24 hours after injection of Ly6C^{hi} monocyte progenitor cells) were analyzed by flow cytometry (*J–N*). Mononuclear phagocyte cell populations were identified (*J*): dendritic cells are designated CD11c$^+$,MHC II^{hi} (D), and macrophages CD11c$^+$,MHC II^{lo} (M). These cell populations (D) and (M) were then further subgated to demonstrate expression of CD11b and Ly6C, (*K*) and (*L*), respectively. Cells more in keeping with macrophages are Ly6C^{hi} (*L*), whereas cells more in keeping with dendritic cells are Ly6C^{lo} (*K*). These adoptive transfer studies demonstrate that Ly6C^{hi} "inflammatory" monocyte progenitor cells (CD45.1 cells) are not present in lung 24 hours (or 72 hours, data not shown) after intravenous injection (*M*); (*N*) media control. Data are presented as mean ± SEM. (*A–I*) n = 6–8; (*J–N*) n = 3. *Bar* = 200 μm.

liver fibrosis (42), and it has recently been proposed that it has a role in tissue remodeling and fibrosis (43). Furthermore, serum and BAL fluid levels of YKL-40 have recently been found to be elevated in patients with IPF versus normal control subjects (44).

We have demonstrated that CD163$^+$ alternatively activated lung macrophages are present in patients with IPF. This fits with recent work showing that CD163$^+$ monocytes are increased in the circulation of patients with progressive IPF compared with normal control subjects (45).

We have for the first time demonstrated the importance of Ly6C^{hi} "inflammatory" monocytes during the progressive fibrotic phase of bleomycin-induced pulmonary fibrosis. Interestingly,

recent work in noninjured mice has shown that Ly6C^{hi} monocytes show up-regulation in gene expression of *Ym1* (*chitinase 3–like protein 3*) (46). With this in mind it is tempting to speculate that Ly6C^{hi} monocytes may be the precursors of lung alternatively activated macrophages, particularly because it has recently been suggested that Ly6C^{hi} monocytes may be the precursors of circulating fibrocytes (47), which have been shown to be important in pulmonary fibrosis (48). However, when we adoptively transferred bone marrow–derived Ly6C^{hi} monocyte progenitor cells from CD45.1 mice into CD45.2 mice, we did not detect CD45.1^{+} Ly6C^{hi} cells within the lungs of the recipient mice, therefore refuting a direct precursor relationship of the Ly6C^{hi} monocytes to the alternatively activated lung macrophages. Some authors suggest that in tissue, Ym1-positive macrophages are derived from Ly6C^{lo} monocytes (49). We did find a population of Ly6C^{hi} mononuclear phagocytes within the lung whose phenotype was more in keeping with a macrophage than a dendritic cell. Following adoptive transfer of Ly6C^{hi} monocyte progenitor cells there was an increase in the proportion of Ym1-positive macrophages within the lung, although in this experimental setting they are not derived from the Ly6C^{hi} circulating monocyte pool. It is important to state that the Ly6C^{hi} cells that were adoptively transferred were not circulating Ly6C^{hi} monocytes *per se*, but were bone marrow–derived Ly6C^{hi} monocytes (monocyte progenitor cells) that may conceivably behave differently from circulating monocytes.

There are several potential mechanisms through which monocytes and macrophages may promote fibrogenesis. During lung fibrogenesis it is possible that macrophages release soluble factors that are important for both myofibroblast survival and epithelial activation. It has been demonstrated that culture supernatants of alveolar macrophages from patients with IPF increased collagen production by normal lung fibroblasts partly mediated via CCL18, a marker of alternative macrophage activation (10). Another candidate soluble factor promoting pulmonary fibrosis is insulin-like growth factor-1. Secretion of insulin-like growth factor-1 by alternatively activated macrophages protects myofibroblasts from apoptosis (50). The importance of TGF-β as a master regulator of fibrosis is well described (35). It has been demonstrated that alveolar macrophages from patients with IPF, but not from normal patients, secrete active TGF-β and are likely to be its major source (12). Interestingly, a study of patients with IPF using *in situ* hybridization demonstrated that $TGF\beta_1$ mRNA was present in macrophages adjacent to fibroblastic foci, but that the foci themselves showed no signal for $TGF\beta_1$ mRNA (51).

We suggest that in patients with IPF the initial insult results from interactions between not only epithelia and fibroblasts, but through a much more complicated and concerted interplay between alternatively activated macrophages, epithelium, and fibroblasts and myofibroblasts. We propose that before and as the fibroblastic focus is being set up as the driver of matrix production, circulating monocytes and fibrocytes traffic to the lungs and promote the formation of alternatively activated lung macrophages that surround and feed this cellular hotspot with a variety of soluble mediators, engage in cell–cell interaction, and promote pulmonary fibrosis. We do not believe our findings detract from prevailing hypotheses regarding the pathogenesis of pulmonary fibrosis but believe they add a further layer of complexity.

One of the most intriguing aspects of our work are the data suggesting that during the reversible phase of bleomycin-induced pulmonary fibrosis, lung macrophage depletion slows fibrosis resolution. It is clear that further work is required to examine this in more detail, because understanding the resolution phase of bleomycin-induced lung fibrosis may provide crucial insights into fibrosis resolution and aid in the development of new therapies.

Importantly, our described importance of alternatively activated macrophages during lung fibrogenesis may provide a scientific rationale for the lack of benefit of corticosteroids in treating fibrotic lung conditions, suggests they may actually exacerbate the problem, may affect clinicians' management of these conditions, and has wider appeal therapeutically for the management of many tissue fibrotic conditions. By enhancing our knowledge of pulmonary fibrogenesis and fibrosis resolution we may enable new molecular, cellular, and pharmaceutical therapeutic approaches to be developed.

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